Survival of Lactobacillus reuteri UBLRu-87 during passage through the unique in vitro gut model system

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Research Article

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Abstract

In this study, the survival of *Lactobacillus reuteri* UBLRu-87 during passage through the unique *in vitro* gut model system was investigated. The viability of strain with and without glucose in GIT was determined by using pour plate and flow cytometer analysis. Lactic acid (D- and L-) production and residual glucose levels were measured as indicators of metabolic activity. In results, as per the plate count method, bacterial survival remained 100 % at 2 h of stomach phase (with and without glucose). Later at 3 h of intestinal phase, it reduced to 52 % with glucose and 100 % without glucose. At 24 h of colonic incubation, viability increased to 143 %, where bacteria utilized ~ 54% of glucose and produced 7.73 ± 0.70 mmol/L L-lactic acid and 0.22 ± 0.01 mmol/L D-lactic acid. In the absence of glucose, strain count was increased from zero to 4.95 ± 0.10 $\log_{10}$ CFU/mL at 24 h of colon incubation. In flow cytometer analysis, the survival of strain at 2 h of stomach phase was 109 % with glucose and 105 % without glucose. Later, it reduced to 74 % at 3 h of the intestinal phase (with and without glucose). At the end of the 24 h colonic incubation, the bacterial viability was increased to 111 % with glucose and 108 % without glucose. In conclusion, under harsh intestinal conditions without glucose, UBLRu-87 cells lose their ability to grow and multiply, but keep a viable but non-culturable (VBNC) state. After favorable conditions in colon, strain VBNC state resuscitate to culturable state. Besides this, glucose favors strain protection and viability enhancing effects during passage of strain throughout the GIT.

Introduction

Probiotics are “live microorganisms that, when administered in adequate amounts, confer a health benefit on the host” [1]. The term “adequate amount” is not properly defined yet, to date, several clinical investigations claim 10^8 to 10^{10} colony forming units (CFU) as effective dose [2, 3]. However, doses may vary from strain to strain and health indications. Besides viability at storage, the information on survival of probiotic bacteria during gastrointestinal tract (GIT) passage is highly important to make dose claims of finished probiotic products. Until now, several studies have reported GIT survival of bacteria by individual gastric and intestinal tolerance tests or unceasingly by suspending cell pellets from gastric to intestinal experiments [4, 5]. However, the behavior of probiotic bacteria in continues *in vitro* simulated gut model systems remains under reported [6, 7].

Over the last few decades, several different GIT models have been developed by the researchers. These models mainly focus on planktonic cells in fed or fasted conditions and operated in batch, continuers or semi-continues modes [6]. Simulator of Human Intestinal Microbial Ecosystem (SHIME) [8] and TNO Gastro-Intestinal Model (TIM) [9] are the well-established GIT models recognized for quality outcomes. Besides this, the cost to study bacterial behavior in such models is high and depends on the number of parameters to investigate. In this study, we used unique in house GIT system to evaluate the behavior of probiotic bacteria. It consists of a series of 3 fully automated reactors (7 L capacity) equipped with pH, and temperature sensors, and other essential accessories to simulate stomach, small intestine and colon conditions (Fig. 1). The composition of gastric, intestinal and colonic fluid is derived from previous

studies to mimic the gut environment [10–12]. As like other *in vitro* GIT models, our unique gut model has no realistic physiological colon environment.

Lactobacillus reuteri UBLRu-87 reported in this study is a well-characterized [13] probiotic strain with proven clinical efficacy in the management of chronic periodontitis [14]. Besides this, along with other strains, it is reported to alleviate bacterial vaginosis [15] and for *in vitro* anticancer activity [16]. In this study, we report on survival behavior of *L. reuteri* UBLRu-87 in unique *in vitro* gut model system with and without glucose. The survival was determined using plate count and flow cytometer analysis. In addition, D-, L- lactic acid production was investigated during short term colonic incubations.

Materials And Methods

Bacteria

L. reuteri UBLRu-87 (MTCC 5403) was obtained from Centre for Research and Development, Unique Biotech Limited, Hyderabad.

Unique Gastrointestinal Tract System

The unique gastrointestinal tract (GIT) system consists of three reactors of maximum 4 liters working capacity. All reactors were equipped with pH and temperature sensors, and fully-automated controller system to simulate conditions of stomach, small intestine and colon (Fig. 1). Behavior of probiotic in GIT was determined by adding strain into the stomach reactor consisting gastric juice for specified incubation time and transferred immediately to the intestine reactor consisting ingredients of intestinal fluid. After intestinal phase incubation, the content was transferred to colon reactor consisting ingredients of colon. The incubation time and pH of the reactors were maintained to mimic *in vivo* GIT conditions. In this study, the behavior of UBLRu-87 was investigated in the presence or absence of glucose in the unique GIT system.

Stomach Phase

The stomach reactor contains gastric juice (consisting: glucose, 2.0 g; proteose peptone, 8.3; KH_2PO_4, 0.6 g; NaCl, 2.05 g; KCl, 0.37 g; $CaCl_2$, 0.11 g; bile, 0.05 g; lysozyme, 0.1 g; pepsin, 0.0133 g; water 1L) [10] of pH 6.8. Immediately after inoculation of bacteria, the pH of the gastric juice was reduced sigmoidal from 6.8 to 2.2 [11]. During this phase, reactor temperature was maintained to 37 ^{o}C and content was stirred at 100 rpm. The samples were withdrawn at 0, 1 and 2 h time intervals where the pH of the content was equal to 6.8 ± 0.1, 3.5 ± 0.1 and 2.2 ± 0.2, respectively. In another experiment, the behavior of UBLRu-87 was investigated in the absence of glucose in gastric juice. The other conditions remained same as described earlier.

Intestinal Phase

The intestinal phase contains 0.1% (w/v) pancreatin (amylase 100 U/mg; lipase 8 U/ mg; protease 100 U/mg) and 0.3% bile (w/v) [12]. Immediately after transfer of stomach content to intestine, the pH was adjusted to 5.5. The content was stirred as described earlier and pH was further reduced to 7.6 at 37 ^{o}C. The samples were withdrawn at 1, 2 and 3 h time intervals where the pH of the content was equal to 6.0 ± 0.2, 7.0 ± 0.1 and 7.6 ± 0.2, respectively. In another experiment, culture in gastric juice without glucose was transferred to intestine reactor and investigated as described earlier.

Colon Phase

The colonic phase incubation was started after transfer of intestinal phase content to colon reactor containing 0.01% (w/v) purified mucin. The pH of the content was adjusted to 8.0 and temperature was maintained to 37 ^{o}C. Nitrogen gas was purged (at 1 h interval) through the colonic content to maintain anaerobic conditions. The samples were withdrawn at 0 and 24 of time intervals and analyzed for viability, residual glucose, and lactic acid production. In another experiment, culture in intestinal phase without glucose was transferred to colon reactor and investigated as described earlier.

Sample Analysis

Pour Plate Method

The samples were each serially diluted in 0.85% (w/v) saline and appropriate dilutions were mixed separately into molten deMan, Rogosa and Sharpe (MRS) agar and poured into the sterile petri-plates. The plates were incubated anaerobically (Forma Steri-Cycle CO_2 incubator, Thermo Fisher Scientific, Massachusetts, USA) at 37 ^{o}C for 24 h. The colonies were counted and results were expressed in $\log_{10}$ CFU/mL.

Flow Cytometer

The samples were centrifuged at 11,000 ×g for 10 min at 4 ^{o}C (Sorvall Legend XTR, Thermo Scientific, USA) and washed with 0.85% (w/v) saline to reduce noise during the analysis. The cells were diluted serially in saline and appropriate dilutions were stained with LIVE/DEAD™ *Bac*Light™ bacterial viability kit (Thermo Scientific, USA). The bacterial viability was analyzed on CytoFLEX flow cytometer (Beckman Coulter, Indianapolis, USA) with slow flow rate (10 µL/min) [17]. The results were expressed as mean $\log_{10}$ CFU/mL

Determination Of Lactic Acid

The samples were centrifuged at 11,000 ×g for 10 min at 4 ^{O}C and the amount of D- and L- lactic acid present in the supernatant was measured by using a NZY Tech, D-/L-lactic acid kit (Lisboa, Portugal), according to the manufacturer's instructions.

Determination Of Glucose

The amount of residual glucose in the samples was determined by using a LabAssay™ Glucose (Mutarotase-GOD method) kit (FujiFilm, Gunma, Japan), according to the manufacturer's instructions.

Statistical Analysis

GraphPad Prism (San Diego, California, USA) was used to perform significant differences between means by Tukey's test after analysis of variance (ANOVA) and *t* -test. The *p*-value < 0.05 was considered statistically significant.

Results

Survival of L. reuteri **UBLRu-87 in Unique Gut Model**

Stomach Phase

Bacterial survival determined by pour plate method showed no significant ($p > 0.05$) changes in viability of UBLRu-87 during stomach phase with glucose (0 h, 7.12 ± 0.05; 1 h, 7.18 ± 0.06; 2 h, 7.13 ± 0.03 $\log_{10}$ CFU/mL) and without glucose (0 h, 6.99 ± 0.02; 1 h, 7.09 ± 0.03; 2 h, 7.13 ± 0.04 $\log_{10}$ CFU/mL) (Fig. 2). Moreover, the viability of UBLRu-87 in gastric juice with and without glucose during 1st and 2nd h of stomach incubation remained same ($p > 0.05$) (Fig. 2).

On the contrary, the survival determined by flow cytometer showed significant increase in viability of UBLRu-87 during gastric phase with glucose (0 h, 7.07 ± 0.07; 1 h, 7.41 ± 0.01; 2 h, 7.71 ± 0.01 $\log_{10}$ CFU/mL) and without glucose (0 h, 7.07 ± 0.03; 1 h, 7.20 ± 0.01; 2 h, 7.44 ± 0.01 $\log_{10}$ CFU/mL) (Fig. 3a, b). Besides this, UBLRu-87 viable count determined at 1st and 2nd h of stomach incubation with glucose was significantly higher (1 h, p 0.002; 2 h, $p < 0.0001$) as compared to without glucose (Fig. 3a).

Intestinal Phase

During the 3 h intestinal phase, the viable count determined by pour plate method showed that the survival of UBLRu-87 transferred along with glucose containing gastric juice was significantly ($p < 0.0001$) reduced from 7.13 ± 0.03 (0 h) to 3.69 ± 0.01 (3 h) $\log_{10}$ CFU/mL (1 h, 4.39 ± 0.08; 2 h, 3.84 ± 0.06 $\log_{10}$ CFU/mL) (Fig. 2). Moreover, UBLRu-87 transferred along with gastric juice without glucose showed

no survival within an hour of intestinal phase incubation (0 h: 7.13 ± 0.04; 1, 2 and 3 h: 0 $\log_{10}$ CFU/mL) (Fig. 2). The viability differences of UBLRu-87 incubated with and without glucose in intestinal phase are significant (*p* 0.0001).

In flow cytometer analysis, UBLRu-87 cells incubated in glucose and without glucose were decreased significantly ($p < 0.0001$) from start to end of the intestinal phase (Fig. 3a). The count in glucose containing intestinal phase was reduced from 7.71 ± 0.01 (0 h), to 6.38 ± 0.06 (1 h), 6.00 ± 0.10 (2 h), and 5.69 ± 0.08 (3 h) $\log_{10}$ CFU/mL (Fig. 3a, c). On the contrary, UBLRu-87 count in intestinal phase without glucose was reduced from 7.44 ± 0.01 (0 h) to 5.30 ± 0.19 (1 h) $\log_{10}$ CFU/mL, and then increased to 5.69 ± 0.08 (2 h) $\log_{10}$ CFU/mL. Later, the phase was ended with the count of 5.49 ± 0.19 $\log_{10}$ CFU/mL (Fig. 3a). Furthermore, the viability of UBLRu-87 incubated with glucose was significantly higher for 1st (*p* 0.0065) and 2nd h (*p* 0.0181) of intestinal phase as compared with UBLRu-87 incubated without glucose. At end of the intestinal phase, cells incubated with and without glucose were ended with no significant (*p* 0.217) difference in viability.

Short Colon Phase

UBLRu-87 viability in presence of glucose was significantly (*p* 0.0003) increased from 0 h (3.69 ± 0.01 $\log_{10}$ CFU/mL) to 24 h (5.28 ± 0.05 $\log_{10}$ CFU/mL) of colonic incubation (Fig. 2). Similarly, the strain incubated without glucose was changed significantly ($p < 0.0001$) from zero pour plate count to 4.95 ± 0.10 $\log_{10}$ CFU/mL at 24 h of colonic incubation (Fig. 2). At end of the colon phase, cells incubated with glucose were significantly (*p* 0.014) higher as compared to cells incubated without glucose.

The samples analyzed on flow cytometer showed that, the viable count of strain incubated with glucose was increased significantly ($p < 0.001$) form 0 h (5.69 ± 0.08 $\log_{10}$ CFU/mL) to 24 h (6.34 ± 0.03 $\log_{10}$ CFU/mL) of colonic incubation (Fig. 3a, d). On the contrary, there were no changes in viability (0 h, 5.49 ± 0.19; 24 h, 5.95 ± 0.04 $\log_{10}$ CFU/mL) of strain incubated without glucose. Besides this, at the end of colon phase, the viable count difference recorded between glucose and without glucose was significant (*p* 0.0005).

Determination Of Lactic Acid

Strain produced 7.73 ± 0.70 mmol/L L-lactic acid and 0.22 ± 0.01 mmol/L D-lactic acid at 24 h of colonic incubation with glucose.

Determination Of Glucose

Glucose levels were remained unchanged in both stomach and intestinal phase incubation with UBLRu-87. However, strain utilized ~ 54% of glucose (residual glucose: 0 h, 163.54 ± 3.87 mg/dL; 24 h, 75.06 ±

1.53 mg/dL) at 24 h of colonic incubation.

Discussion

The survival of probiotic bacteria in gastrointestinal tract (GIT) is crucial to impart intended health benefits to the host. Studies evaluating *in vitro* probiotic properties like gastric and intestinal tolerance were either investigated independently and or unceasingly by suspending cell pellets from gastric to intestinal experiments. Besides this, the outcome of such investigations do not provide possible real-time behavior of probiotic strains in GIT. In order to understand bacterial transit through GIT, in this study we used a unified fully automated reactors to mimic stomach, intestine and colon conditions, and investigated survival of *L. reuteri* UBLRu-87 in presence or absence of glucose by using plate count and flow cytometer method. Lactic acid production and glucose utilization pattern was studied for culture with-glucose during short phase colonic incubation.

After ingestion, probiotic bacteria need to survive under acidic stomach conditions and the detrimental action of gastric enzymes [18]. In this study, the viability of UBLRu-87 in stomach reactor with and without glucose was not affected significantly could be due to a sigmoidal decrease of gastric juice pH (0 h: 6.8 to 2 h: 2.2), which may allowed cells to get prepare against gastric stress. Besides this, in flow cytometer analysis, cells with glucose showed significantly higher survival as compared with cells without glucose, suggesting a protective and growth-enhancing effect of glucose in acidic conditions. Though, the cells detected by flow cytometer are viable but non-culturable (VBNC), they have a propensity to resuscitate under favorable conditions. These results corroborate well with a previous finding that glucose protects and enhances survival of lactobacillus under acidic conditions could be due to up-regulating F_0F_1-ATPase activity [19]. Furthermore, no change in residual glucose during the stomach phase suggested cells may have utilized glucose at trace levels which remained undetected. In general, pre-adaptation, cross-protection, protection of macromolecules, up regulation of F_0F_1-ATPase activity, amino acid decarboxylation, and production of alkaline compounds in the cytoplasm are reported as acid resistance mechanisms in lactic acid bacteria [20].

After the stomach, bacteria reaches the intestine, where survival in the presence of bile and pancreatic enzymes is more challenging. In this study, UBLRu-87 viable plate count was reduced to 38% at 1 h and, 46% and 48% during subsequent hours of intestinal incubation with glucose. Whereas no viable cells were detected when strain incubated without glucose. On the contrary, flow cytometer analysis showed a 17% reduction of viability at 1 h and, 22% and 26% during subsequent hours of intestinal incubation with glucose. Whereas, the viability of cells without glucose was reduced to 28% at 1 h and later became comparable (2 h, 23%; 3 h, 26%) with glucose phase. These results suggested that glucose may protect cells by an unknown mechanism and or bile salt hydrolase and bacteria resistant to pancreatic enzymes may be responsible for improving survival under intestinal conditions [21]. Besides this, the higher cell numbers determined by flow cytometer as compared with plate count method is indicative of cells VBNC state. However, such cells have a propensity to resuscitate under favorable conditions. Moreover, as like stomach phase, changes in residual glucose levels remained undetected in the intestinal phase.

During the short 24 h colonic incubation, UBLRu-87 cells utilized ~ 54% glucose and produced both L- and D- enantiomers of lactic acid. This reduced the pH of the colon reactor from 8.0 to 6.4 units. The production of lactic acid in the colon is known to favor the growth of other lactic acid bacteria and helps to restore healthy microbiota composition [22]. The increased viable count of UBLRu-87 during colonic incubations with and without glucose indicated cells ability to restore multiplication potential and metabolic activity. It is interesting to note that cells without glucose were resuscitated from VBNC state to viable culturable cells, which could be due to the mucin in the colon reactor. The pH of the colon reactor without glucose decreased from 8.0 to 7.7 units at 24 h of colonic incubation. However, the metabolites produced in colon reactor without glucose were not investigated in the present study. Overall, based on plate count and flow cytometer analysis, glucose protects cells to retain a viable and culturable state throughout the GIT journey.

Conclusion

In conclusion, the survival of *L. reuteri* UBLRu-87 was decreased drastically during intestinal passage and improved at short colonic incubation. UBLRu-87 produced both D- and L- lactic acid from glucose and was capable of reducing colon pH. Based on plate count and flow cytometer analysis, glucose enhanced viability and protected cells to retain a culturable state throughout the GIT journey. Moreover, we recommend plate count coupled with flow cytometer analysis to understand the behavior of bacteria through an *in vitro* gut model system.

Declarations

Author Contributions

Ahire JJ contributed to the study conception and design. Material preparation, data collection and analysis were performed by Ahire JJ, Mokashe NU, and Kashikar MS. The first draft of the manuscript was written by Ahire JJ. Madempudi RS revised the manuscript critically. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Data Availability

All data is included in the text, however, the raw data of this article will be made available by the authors, without undue reservation, to any qualified researcher.

Ethics approval

This study does not contain any work related with participation of humans and/or animals.

Competing Interests

Ahire JJ, Mokashe NU, and Kashikar MS were employed by Unique Biotech Limited. Madempudi RS is the Managing Director of Unique Biotech Limited. This does not alter our adherence to journal policies on sharing data and materials.

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Figures

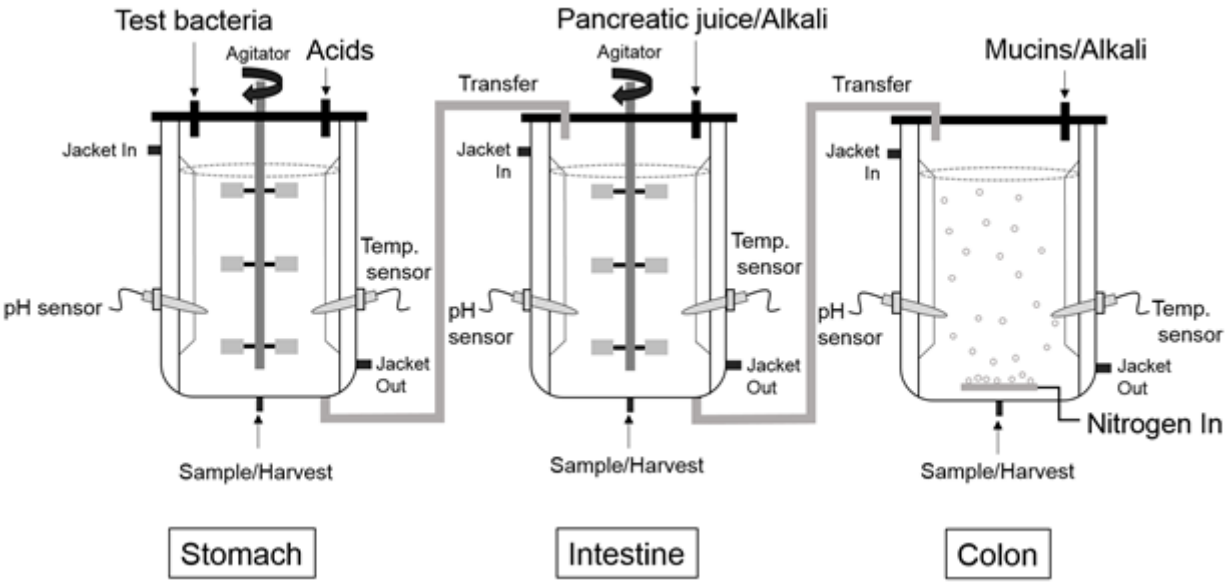

Figure 1

Schematic representation of a unified reactors of unique *in vitro* gut model system

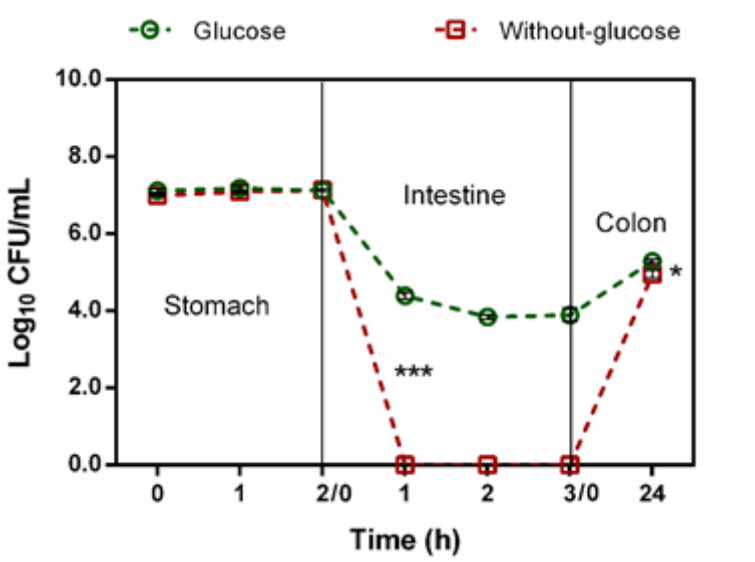

Figure 2

Survival of *Lactobacillus reuteri* UBLRu-87 in unique *in vitro* gut model system with and without glucose. The $\log_{10}$ CFU/mL determined by pour plate method. All data are represented as mean ± SD. *** $p \leq 0.001$ and * $p \leq 0.05$.

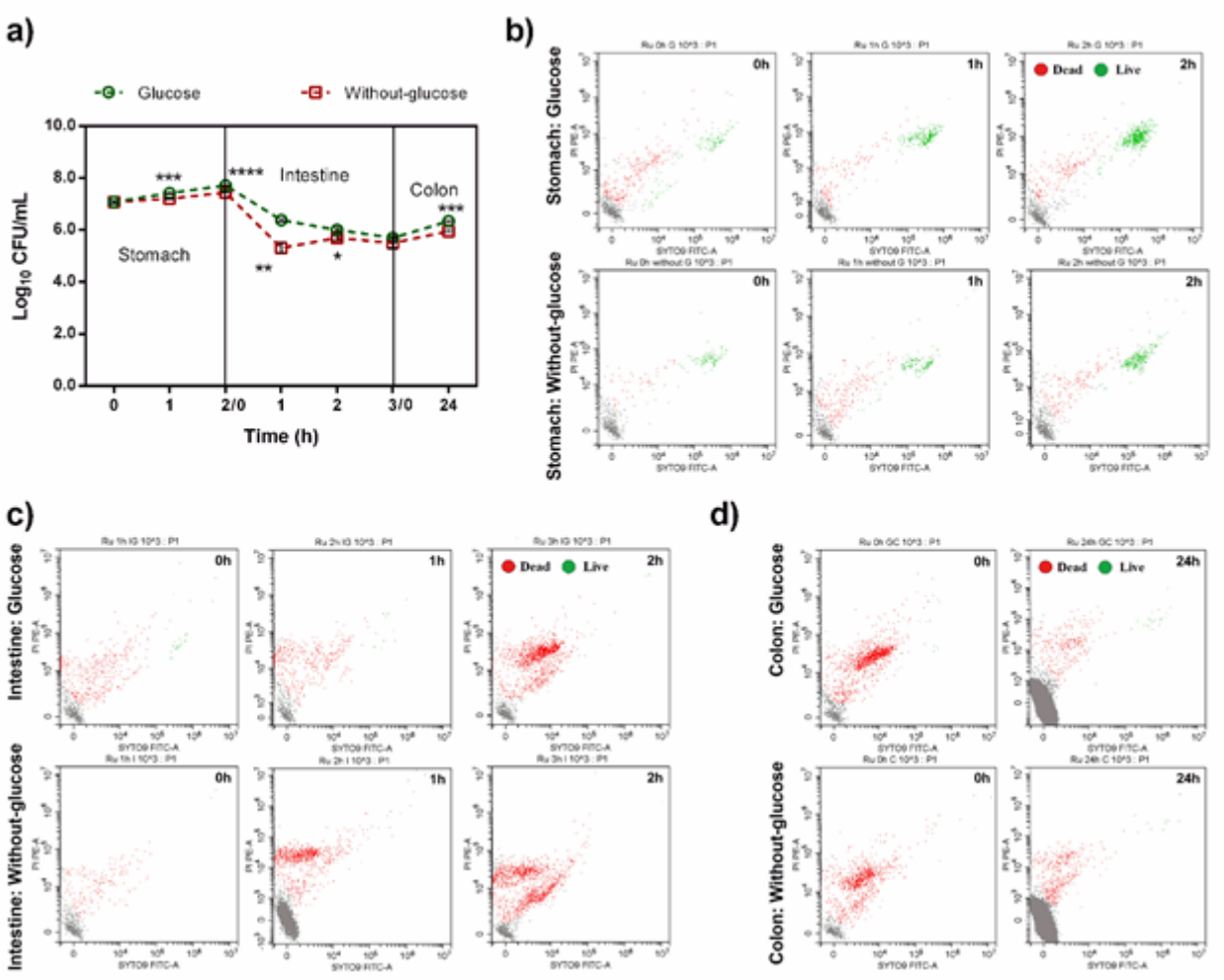

Figure 3

Survival of *Lactobacillus reuteri* UBLRu-87 in unique *in vitro* gut model system with and without glucose. Log_{10} CFU/mL determined by flow cytometer (a); viable cell events in stomach (b), intestine (c), and colon (d). **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, and * $p \leq 0.05$.